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Immunization Against Bacteria- and Endotoxin-Induced Hypotension

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The characteristics of hypotension induced in rabbits by intravenous injection of viable *Salmonella typhosa* O901 organisms were studied and found to be a function of the number and age of the bacterial cells. Effective neutralization of the blood pressure lowering was achieved by immunization with homologous organisms as well as heterologous endotoxins and a detoxified derivative. In addition, native endotoxins derived from a number of different genera of gram-negative bacilli, as well as lipopolysaccharides deficient in either the polysaccharide or lipid components, were tested for their ability to induce hypotension in rabbits and tolerance to the lowering of blood pressure. Hypotension was elicited by intravenous injection of all native endotoxins as well as polysaccharide-deficient endotoxins, but was absent in preparations from which the lipid was removed. On the other hand, protection against the hypotension effect could be induced after injection of either the lipid- or polysaccharide-deficient derivatives.

Active and passive immunization against the multifaceted toxicity of gram-negative bacteria and their endotoxin component by antibacterial and anti-lipid A antiserum is of interest in view of the failure of drug therapy to alleviate sepsis and the high death rate. The major findings in this area have been summarized (7) and suggest that a protective antigen lies in the lipid A, 2-keto-3-deoxyoctonic acid (KDO) and polyheptose phosphate complex.

Much of the early experimental data have been acquired in dogs; however, this species, in contrast to humans, requires large amounts of endotoxin for hypotensive action, and reproducibility of results has been difficult (17). On the other hand, the febrile response, the production of tolerance to pyrogenicity, the antibody response, and the production of peripheral leucocytosis in rabbits to purified endotoxins have been found to be similar in dogs and humans (9). Accordingly, we used a sensitive system developed earlier in our laboratory with the rabbit as the experimental model (A. G. Johnson and A. Anderson-Imbert, Bacteriol. Proc. 1965) to (i) characterize the hypotensive action of a representative gram-negative bacillus, *Salmonella typhosa* O901, and (ii) extend this system in a study of various native endotoxins (NE), as well as those deficient in either the polysaccharide or lipid structural components, to determine their hypotensive effect and the capacity of each to protect against this important endotoxin characteristic.

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MATERIALS AND METHODS

Blood pressure assays. Twelve- to 14-week-old Dutch Belt rabbits were anesthetized locally by injecting 3 ml of Seracaine (lidocaine hydrochloride, 1%) intramuscularly and subcutaneously in the area of the femoral artery. The latter was exposed, cannulated, and connected to a manometer which recorded the blood pressure on a kymograph. One milliliter of heparin (10,000 USP units) was injected into this artery to prevent clotting. After the blood pressure had stabilized, the preparation to be tested was injected intravenously (i.v.), and the blood pressure was recorded at 15-min intervals for 2 h. Gauze soaked with the anesthetic was placed on the incised area during the 2-h period. The selection of local rather than systemic anesthesia was found to be important since the latter resulted in an unstable normotensive pressure in this species. For each preparation tested, 5 to 10 rabbits were used, unless recorded otherwise. All immunized rabbits were challenged with 7×10^8 viable *S. typhosa* O901, unless noted otherwise.

Organisms. *S. typhosa* strain O901 was obtained from A. Abrams, Walter Reed Army Medical Center, Washington, D.C., and cultured in Trypticase soy broth (BBL Microbiology Systems) under the conditions indicated below. Antibody titers were determined by the O-agglutinin assay.

Endotoxins. The lipopolysaccharides (LPS) and products used in this study were prepared by O. Luderitz, Max Planck Institut für Immunobiologie, Freiburg, West Germany, except for that from *Serratia marcescens* and the polysaccharide designated Ps,

which were prepared by one of us as described previously (15). Unless otherwise specified, all were suspended in 0.15 M NaCl to the desired concentrations.

(i) NE preparations. NE were extracted by the phenol-water procedure (20) from smooth strains of *S. minnesota* (S99), *S. typhimurium*, *S. uganda*, and *S. godesberg* and from a rough strain of *Escherichia coli* B/r. Except for the endotoxin isolated from *E. coli*, they consisted of O-specific side chains, the basal core, and lipid A. The *E. coli* B/r endotoxin consisted only of part of the basal core (glucose, heptose, KDO, ethanolamine, and lipid A); the O-specific side chains were absent.

(ii) Polysaccharide-deficient endotoxins (PDE). The Re LPS were extracted by the phenol-water procedure (20) from rough mutants of *S. minnesota* (R595), *S. typhimurium* GiI), and *S. godesberg*. Since these mutants lack the enzymes required to synthesize heptose or the transferases required for the addition of heptose onto the growing polysaccharide, the LPS consisted of KDO, ethanolamine, and lipid A only.

(iii) Lipid-deficient endotoxins (LDE). These were prepared by growing the amoeba *Dictyostelium discoideum* on *S. london* or *E. coli* B/r and then extracting the amoeba by the phenol-water procedure (10, 11). The resulting preparations were further purified by ethanol precipitation. The *E. coli* B/r preparation consisted of glucose, heptose, KDO, phosphate, ethanolamine, and glucosamine. The Ps preparation was obtained from the supernatant after hydrolysis in 1 N HCl of *Serratia marcescens* endotoxin. It contained no measurable fatty acids and no KDO (15).

(iv) Detoxin. Detoxin was a pyridinium formate-treated endotoxin from *S. marcescens* which exhibited a decrease in short chain *O*-acyl linkages (14).

Protection induced by LDE. Initially, 100 μg of LDE was given i.v., and 6 days later rabbits were prepared for blood pressure assays and challenged with 100 μg of NE. In the second procedure, rabbits were given 50 μg of LDE i.v. on day 0 and another 50 μg on day 6. Twenty-four hours after the last injection, the rabbits were prepared for blood pressure assays and challenged i.v. with either 100 μg of the NE from which the LDE was derived or NE preparations isolated from other bacterial genera.

Protection induced by PDE. On day 0, 12- to 14-week-old Dutch Belt rabbits were injected i.v. with 10 μg of Re LPS (PDE). On days 3 and 6, another 10 μg was injected. Twenty-four hours after the last injection, the rabbits were prepared for blood pressure assays and challenged i.v. with 100 μg of the NE preparation from which the PDE was derived or NE from other bacterial genera.

RESULTS

Induction of hypotension. In initial experiments, the number of viable *S. typhosa* cells necessary to produce hypotension after i.v. injection into rabbits was tested and found to be reasonably proportional to the number of cells injected. Noteworthy was the observation that a latent period of 10 min or greater occurred invariably in each rabbit before the initial decrease in blood pressure. Since 7×10^8 cells produced routinely a drop in blood pressure of greater than 20 mm of Hg, this concentration was chosen for challenge in the majority of experiments. In a typical experiment using this concentration in three rabbits, the individual drops in blood pressure were 24, 30, and 44 mm of Hg. When the effect of the age of the bacterial cells was tested, it was found that a concentration of 7×10^8 cells grown for 48 h was much less capable of inducing hypotension than this same concentration of cells grown for 14 to 18 h (Fig. 1).

Immunization against bacteria-induced hypotension. In preliminary experiments to determine whether rabbits could be actively immunized against the hypotension induced by viable *S. typhosa* O901, rabbits were injected i.v. three times at 3-day intervals with 50 μg of acetone-killed and dried (AKD) *S. typhosa* O901 cells and challenged with viable bacilli 7 days after the last injection. It was found that the drop in blood pressure could be prevented by this immunization regimen.

To determine how rapidly this specific immunity would develop, and its duration, rabbits were immunized with AKD cells as above and bled, and individual animals were challenged with the standard dose of 7×10^8 cells on days 1, 6 to 8, 12 to 14, 20, 30, and 35 after the last

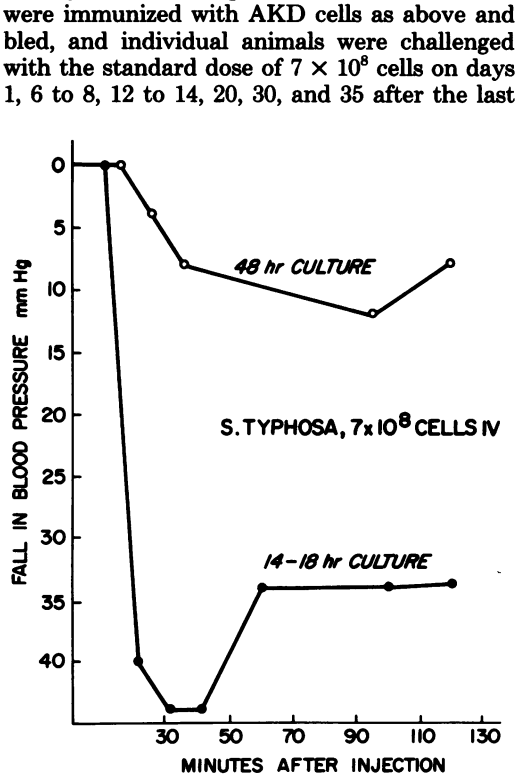

FIG. 1. *Effect of age of S. typhosa O901 cells on ability to induce hypotension in rabbits.*

injection. Protection was observed within a day after the last injection and was still present after 14 days (Table 1). By 20 days it had waned, continuing at a lesser degree through the 30th day. By 35 days, protection had essentially disappeared. O-agglutinin titers were the highest during the time of peak protection, but no correlation could be drawn since only a single rabbit was used for each determination.

Figure 2 shows the temporal effect of a single i.v. immunizing dose of 50 μg of AKD cells in protecting against hypotension. The protection afforded was not evident on day 1, was highest on day 4, and had disappeared by day 22. Antibody appeared on day 4 (1:160), reached a peak of 1:1,280 on day 7, and had waned to 1:80 on day 22. A second injection of AKD cells in a separate group of immunized rabbits on day 50 induced absolute protection 1 day later (data not shown). A portion of this latter group challenged at 35 days (15 days before reinjection) showed a blood pressure drop comparable to that of the control (25 mm of Hg), indicating that no protection from the first injection of AKD cells existed at this time.

To determine whether the well-established phenomenon of nonspecific increase in resistance to infection induced by gram-negative endotoxin (16) also might be contributing to protection, the efficacy of an endotoxin from *Serratia marcescens*, an organism putatively unrelated to *Salmonella typhosa*, in protecting rabbits against the hypotensive property induced by viable *S. typhosa* cells was tested. Protection against this parameter of the pathogenicity of gram-negative organisms could be induced by i.v. injection of quantities of 5 μg or greater of a serologically unrelated endotoxin

TABLE 1. *Duration of protection against hypotension after three immunizing injections of S. typhosa O901 (AKD)*

Days after last injection	Peak drop in 2 h (mm of Hg)	Reciprocal of titer antibody
1	6	160
6	6	640
7	0	2,560
8	6	2,560
12	10	1,280
13	6	1,280
14	0	1,280
20	16	320
30	14	320
35	22	320
Control	30[a]	<10
	40	<10
	24	<10
	30	<10

[a] Average control value, 31.

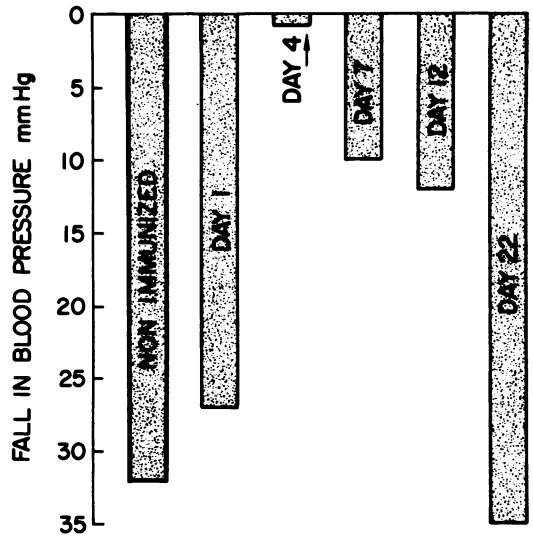

FIG. 2. *Protection against hypotension induced by specific immunization with one injection of killed S. typhosa O901, 50 μg, and challenge with 7 × 10^8 viable S. typhosa.*

(Table 2). In addition, a detoxified derivative of this parent *Serratia* endotoxin (50% lethal dose for Swiss mice raised from 180 to 2,000 μg) was tested (14). The detoxin also was capable of eliciting tolerance, although at a slightly higher dose level (Table 2). A single injection of either the parent endotoxin or the detoxin conferred protection for at least 7 days (Table 3). In the case of the detoxin, this had disappeared by the 15th day. A more durable tolerance lasting at least 17 days, however, was achieved in a single experiment wherein 10 μg of the detoxin was given three times at 3-day intervals (data not shown).

Induction of hypotension by endotoxin and derivatives. The efficacy of NE and their isolated lipid and polysaccharide moieties in reproducing the blood pressure drop described above for the whole bacilli was also tested. Initial experiments established 100 μg as a dosage of NE capable of causing routinely a profound drop of about 30 mm of Hg. However, after exposure of the parent organism (*E. coli*) to the amoeba *D. discoidium* and subsequent isolation of the lipid-deficient material, this property was lost (Fig. 3). The findings were the same in like experiments with *S. london*. Carbohydrate-deficient Re LPS, on the other hand, were fully capable of inducing hypotension, as is shown in Fig. 4, which compares the blood pressure drop of rabbits receiving the parent endotoxin derived from a smooth organism, *S. minnesota* S99, with the drop seen in rabbits given endotoxin from a rough, carbohydrate-deficient organism, *S. min-*

TABLE 2. *Protection against hypotension induced by a single injection of a serologically unrelated endotoxin or its detoxified derivative from S. marcescens*

Immunization with:	Amount (μg)	No. of rabbits	Peak drop in 90 min[a] (mm of Hg)
Endotoxin	1	2	36
			22
			40
Endotoxin	5	3	8
			0
			6
Endotoxin	50	2	8
			4
Detoxin	5	2	24
			34
Detoxin	10	2	10
			0
Detoxin	50	2	4
			3
Detoxin	100	2	4
			2
None		6	31

[a] All rabbits were challenged with 7×10^8 viable *S. typhosa* 1 day after immunization.

TABLE 3. *Duration of protection induced by heterologous detoxified endotoxin against hypotension*

Immunization with:	Amount (μg)	Days before challenge	Peak drop in 90 min[a] (mm of Hg)
Endotoxin	5	1	9
Endotoxin	5	3	0
Endotoxin	5	7	6
Detoxin	5	1	24
Detoxin	5	3	34
Detoxin	5	7	24
Detoxin	10	1	0
Detoxin	10	4	8
Detoxin	10	7	3
Detoxin	10	15	32

[a] All rabbits were challenged with 7×10^8 viable *S. typhosa* 1 day after immunization.

nesota R595, and an LDE from *E. coli*. The behavior of endotoxins from the smooth and rough strains of *S. typhimurium* compared with the lipid-deficient material from *S. london* paralleled and confirmed that seen in Fig. 4.

Induction of neutralization of endotoxin-induced hypotension. A rapid tolerance to several of the biological properties of endotoxin is generally achieved after single or multiple injections of similar or dissimilar (cross-tolerance) endotoxins. The production of tolerance has been related to the production of antiendotoxin antibody (4, 8). To determine whether protection against the hypotension facet of endotoxicity could be induced by prior injection of LDE or PDE, rabbits were injected once with 100 μg of *E. coli* LDE and challenged 1 day later with 100 μg of the NE from this same organism. No protection was observed (Fig. 5). On the

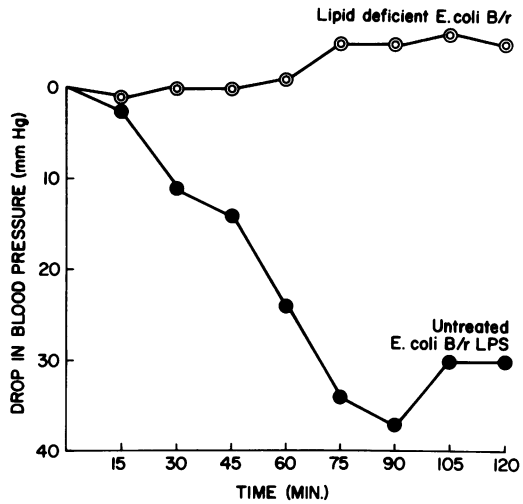

FIG. 3. *Inability to induce hypotension of 100 μg of LDE isolated after exposure of E. coli B/r to the amoeba D. discoidium. The arithmetic mean average of results in five to eight rabbits is plotted.*

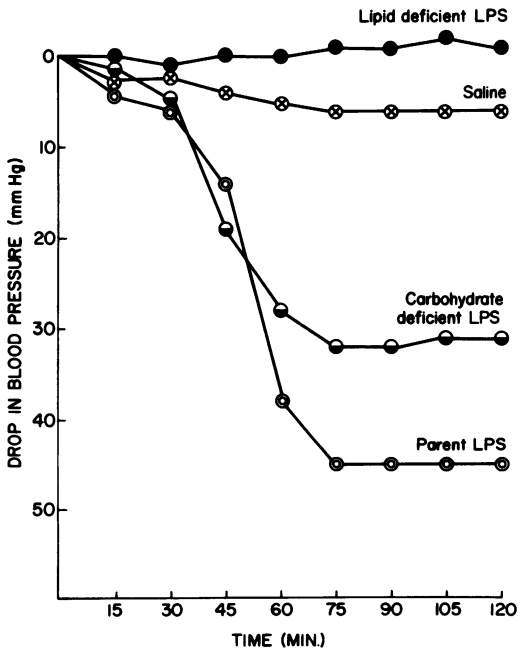

FIG. 4. *Comparison of the hypotension-inducing ability of 100 μg of a lipid-deficient LPS (derived from E. coli) with a carbohydrate-deficient Re LPS and its parent endotoxin (derived from S. minnesota). The arithmetic mean average of results in 6 to 10 rabbits is plotted.*

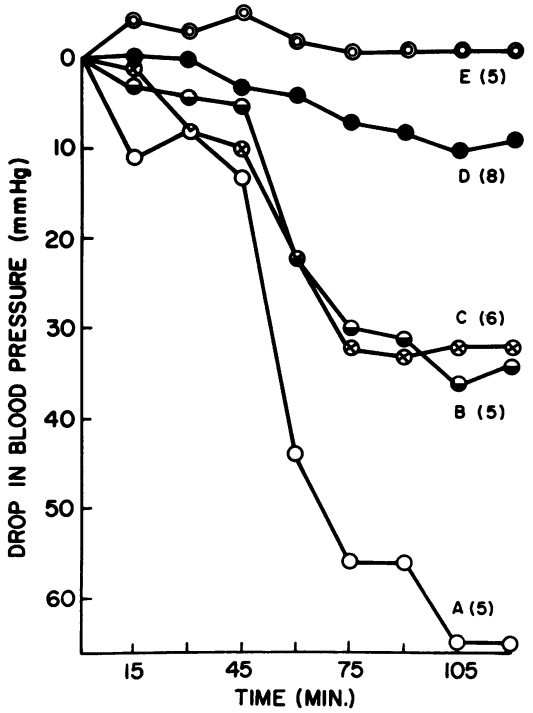

FIG. 5. *Production of tolerance to hypotension with LDE. (A) Rabbits were injected i.v. with 50 μg of E. coli LDE on days 0 and 6 and challenged 24 h later with 100 μg of NE from S. minnesota. (B) Rabbits were injected i.v. once with 100 μg of E. coli LDE and challenged 24 h later with 100 μg of NE from E. coli. (C) Rabbits were injected i.v. once with physiological saline and challenged 24 h later with NE from E. coli. (D) Rabbits were injected i.v. with 50 μg of E. coli LDE on days 0 and 6 and challenged 24 h later with 100 μg of NE from E. coli. (E) Rabbits were injected i.v. with 50 μg of E. coli NE on days 0 and 6 and challenged 24 h later with 100 μg of NE from E. coli. Number of rabbits used is given in parentheses; the arithmetic mean decrease in pressure is plotted.*

other hand, when this dose was divided into two injections of 50 μg of LDE given on days 0 and 6, protection was evident a day later to 100 μg of the NE. However, cross-protection with this regimen was not produced to hypotension induced by endotoxin from a different organism, *S. minnesota.*

Confirmation was achieved when protection against *S. uganda* NE was produced by two like injections of the LDE from *S. london* (Fig. 6), which has a serologically identical O antigen. In addition, several of the biological activities of NE have been found recently to be reproducible by an isolated Ps moiety prepared in a different laboratory with a different method (15). When such a Ps preparation was tested for its capacity to induce protection against hypotension, again two injections on days 0 and 7 of 50 μg of Ps were found to be as effective as the NE (Fig. 7).

Similar experiments with polysaccharide-deficient Re LPS revealed not only a protective effect against the NE, but an effective cross-tolerance to challenge with other organisms (Fig. 8).

DISCUSSION

Since hypotension is the cardinal sign of endotoxin shock, this parameter was used by us to study chemical structure-protective relationships. Earlier we reported the conditions for and the characteristics of hypotension in rabbits after administration of endotoxin (A. M. Abdelnoor and A. G. Johnson, Bacterial Proc., p. 71, 1969; A. M. Abdelnoor, Ph.D. thesis, University of Michigan, Ann Arbor, 1969). A similar system was used by Ulevitch et al. (18) and expanded to show that complement involvement was minimal in endotoxin-induced hypotension. In agreement with the previous findings (13, 18), our results indicate that the lipid portion of the molecule is responsible for the hypotensive effect of endotoxin, since the carbohydrate-deficient R595 preparation was as active as the parent preparation and the lipid-deficient preparations were inactive. Yet, paradoxically, the polysaccharide preparations, similar to the lipid moiety, were fully capable of inducing protection against the hypotension caused by their parent organism. The following hypothesis might be

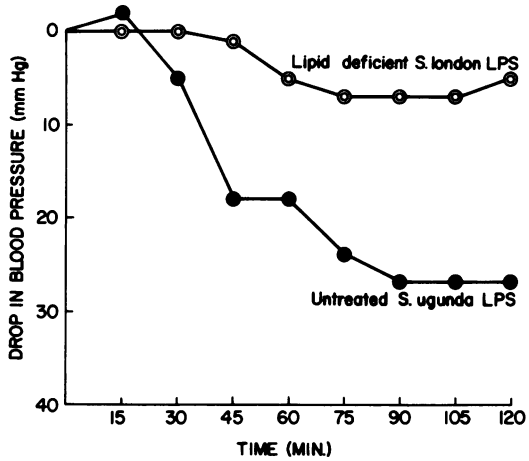

FIG. 6. *Production of tolerance to hypotension with LDE. (A) Rabbits were injected i.v. with 50 μg of S. london LDE on days 0 and 6 and challenged 24 h later with 100 μg of NE from S. uganda. (B) Rabbits were injected i.v. with physiological saline on days 0 and 6 and challenged 24 h later with 100 μg of NE from S. uganda. Number of rabbits used is given in parentheses; the arithmetic mean decrease in pressure is plotted.*

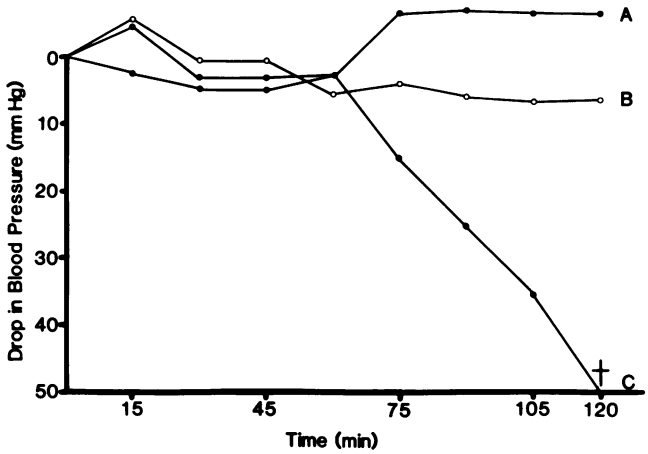

FIG. 7. *Production of tolerance to hypotension with a purified Ps preparation derived from S. marcescens O8. (A) Rabbits were injected i.v. with 50 μg of NE from S. marcescens on days 0 and 7 and challenged 24 h later with 100 μg of NE from S. marcescens. (B) Rabbits were injected with 50 μg of Ps preparation derived from S. marcescens on days 0 and 7 and challenged as in (A). (C) Rabbits were injected with saline and challenged as in (A). The arithmetic mean blood pressure drop of three rabbits in each group is plotted.*

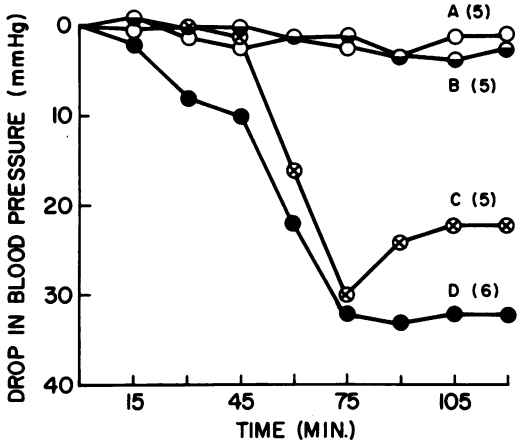

FIG. 8. *Production of tolerance to hypotension with PDE. (A) Rabbits were injected i.v. with 10 μg of PDE from S. typhimurium on days 0, 3, and 6 and challenged 24 h later with 100 μg of NE from E. coli B/r. (B) As in (A), except challenged with NE from S. typhimurium. (C) Rabbits were injected i.v. with saline and challenged as in (B). (D) Saline-injected rabbits were challenged as in (A). Number of rabbits used is given in parentheses; the arithmetic mean decrease in pressure is plotted.*

advanced in explanation. Several different ligands may exist on the lipid moiety, one responsible for signaling the drop in blood pressure, another responsible for eliciting protective tolerance (antibody?). The latter would be shared by both the polysaccharide and the lipid A. The nature (or occurrence) of this group may only be speculated; however, no fatty acids could be detected in the Ps preparation (15), and the extreme acid lability of KDO would probably eliminate KDO as the active, protective ligand under the conditions of Ps isolation. Recently a less acid-sensitive moiety has been found in endotoxin preparations which could be detected in either the lipid or polysaccharide fraction, depending on the conditions of isolation. This may be a logical candidate for the protective ligand (A. Nowotny, unpublished data). Possession of a common determinant group retained by the lipid-deficient as well as the lipid fraction of endotoxin could lead to rapid immunoglobulin M antibody formation, thus neutralizing the challenge dose.

Detoxified derivatives may retain the same ligand or protect by a different mechanism. It has been shown by Golub et al. (5) that chemically detoxified endotoxin will remain in the circulation for a prolonged period of time, whereas toxic endotoxin is removed by the reticuloendothelial system within minutes. If endotoxin-specific receptors on certain cellular elements in the circulation exist, the nontoxic LPS derivative in the circulation could block such receptors and prevent toxic endotoxin injected subsequently from combining and, thus, be rendered ineffective. Such protection against endotoxin lethality in guinea pigs has been elicited with potassium methylate-detoxified (deacylated) LPS pretreatment by Urbaschek and Nowotny (19).

Braude et al. (2) showed that rabbit antiserum to the galactose-deficient mutant (J5) of *E. coli* O:011 prevented death of mice from endotoxin as well as the local generalized Shwartzman reaction. In addition, using a granulocytopenic model of experimental bacteremia developed by them in rabbits (1), antiserum to the core gly-

colipid of gram-negative bacilli was capable of increasing survival rates of rabbits with bacteremia due to *Pseudomonas aeruginosa* and to several strains of *E. coli* and *Klebsiella pneumoniae*. In contrast, antiserum to the parent, *E. coli* O:111, gave no protection, suggesting that the O-side chain sugars may mask the efficacy of the core determinants.

In other studies, McCabe et al. (12) showed that active and passive immunization with the Re chemotype mutants of *S. minnesota* or their antiserum afforded better protection against heterologous gram-negative lethal bacteremia than the smooth strain or its Ra-Rd mutants. Antibody to lipid A per se had no effect. Davis et al. (3) also found that antibody to core glycolipid provided heterologous protection against the dermal necrosis of the local and generalized Shwartzman reaction. On the other hand, Young and Stevens (21) found antiserum to core glycolipid to be less protective of dogs than type-specific immunization. The importance of testing preimmune normal serum concomitantly with immune serum in passive protection studies has been emphasized by the results of Greisman et al. (6). The latter were unable to confirm the conclusion that heterologous antisera to the lipid A core or rough gram-negative bacterial mutants confer broad-spectrum protection to mice against challenge with smooth *Enterobacteriaceae*. In their study, equal protection was achieved with preimmune serum from the same donors.

Our studies extend the above observations in establishing that protection can be achieved against this important parameter of endotoxicity by immunization with detoxified derivatives and the nontoxic polysaccharide portion from homologous and heterologous endotoxins, as well as by smooth bacilli, isolated endotoxins, and the lipid core. Whether protection in each instance involves specific or cross-reacting antibody or is due to some nonspecific mechanism awaits further study.

ACKNOWLEDGMENTS

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